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Horizontal Transfer of Tetracycline Resistance among *Chlamydia* spp. In Vitro▽

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There are no examples of stable tetracycline resistance in clinical strains of *Chlamydia trachomatis*. However, the swine pathogen *Chlamydia suis* is commonly tetracycline resistant, both in America and in Europe. In tested U.S. strains, this resistance is mediated by a genomic island carrying a *tet*(C) allele. In the present study, the ability of *C. suis* to mobilize *tet*(C) into other chlamydial species was examined. Differently antibiotic resistant strains of *C. suis*, *C. trachomatis*, and *Chlamydia muridarum* were used in coculture experiments to select for multiply antibiotic resistant progeny. Coinfection of mammalian cells with a naturally occurring tetracycline-resistant strain of *C. suis* and a *C. muridarum* or *C. trachomatis* strain containing selected mutations encoding rifampin (rifampicin) or ofloxacin resistance readily produced doubly resistant recombinant clones that demonstrated the acquisition of tetracycline resistance. The resistance phenotype in the progeny from a *C. trachomatis* L2/ofl^R–*C. suis* R19/tet^R cross resulted from integration of a 40-kb fragment into a single ribosomal operon of a recipient, leading to a merodiploid structure containing three rRNA operons. In contrast, a cross between *C. suis* R19/tet^R and *C. muridarum* MoPn/ofl^R led to a classical double-crossover event transferring 99 kb of DNA from *C. suis* R19/tet^R into *C. muridarum* MoPn/ofl^R. Tetracycline resistance was also transferred to recent clinical strains of *C. trachomatis*. Successful crosses were not obtained when a rifampin-resistant *Chlamydophila caviae* strain was used as a recipient for crosses with *C. suis* or *C. trachomatis*. These findings provide a platform for further exploration of the biology of horizontal gene transfer in *Chlamydia* while bringing to light potential public health concerns generated by the possibility of acquisition of tetracycline resistance by human chlamydial pathogens.

Members of the chlamydiae are obligately intracellular bacteria that cause serious diseases in a wide variety of hosts (19). In humans, *Chlamydia trachomatis* causes trachoma and a variety of sexually transmitted conditions, diseases that affect millions of people around the world (25). Two related species, *Chlamydia suis* and *Chlamydia muridarum*, cause diseases of mucosal membranes in pigs and mice, respectively. A second genus within the chlamydiae includes the more distantly related guinea pig pathogen *Chlamydophila caviae* and the human pathogen *Chlamydophila pneumoniae*. While the members of the chlamydiae are generally similar in many aspects of basic biology, including genome order and gene content, there are differences in their intracellular survival strategies. One of these differences is the ability to form fusogenic inclusions. Wild-type strains of the *Chlamydia* spp. form intracellular vacuoles (termed inclusions) that undergo homotypic fusion (30), while no tested strains of these species form inclusions that fuse with inclusions of *Chlamydophila caviae* or *Chlamydophila pneumoniae* (23) (see Fig. 1) (unpublished data).

The genome sequences of most chlamydial species do not contain examples of recent horizontal acquisition of DNA. To date, the only reported example of a genomic island within any species of *Chlamydia* is the *tet*(C) island of *C. suis*. This island contains a *C. suis*-specific insertion element (IS*cs605*), plasmid sequence with roots in gram-negative bacteria, and a *tet*(C) resistance gene (10). The tetracycline-resistant strains are common in swine herds in the United States (1) and recently have been identified in pigs from Italy (8). The strains carrying this island are the only examples of naturally acquired antibiotic resistance in any chlamydial species. The *C. suis* tetracycline-resistant strains are also resistant to doxycycline, one of two contemporary front-line drugs of choice against chlamydial infection in humans. Because of its low cost, tetracycline is also used to treat millions of cases of trachoma in developing countries (20). Although there are reports of drug resistance in strains collected from patients suffering treatment failure, documented stable homotypic drug resistance to antibiotics used for treating acute human chlamydial infections is controversial (18, 24, 28). Transfer of a stable tetracycline-resistant phenotype to human clinical chlamydiae would represent a significant public health challenge.

The very limited examples of horizontally acquired DNA in the chlamydiae support a hypothesis that recombination might be rare in this system. However, accumulating sequence data indicate that *Chlamydia* spp. are actively recombinogenic within a species. Early sequencing studies identified *ompA* variants that encode protein sequences from different classical serovars and showed that recombination may also occur at other locations in the chromosome (2, 11, 17, 21). Additionally, recent studies demonstrated conclusively that lateral gene transfer can be selected for in *C. trachomatis* in cell culture following coinfection with strains that have dissimilar drug resistance markers (6, 7). The mechanism of transfer in any of

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TABLE 1. Oligonucleotide primers used in this study

Target	Primer sequence (5′ to 3′)	
	Forward	Reverse
tet(C)	AGCACTGTCCGACCGCTTTG	TCCTCGCCGAAAATGACCC
C. trachomatis L2 *ompA*	CTGTAGCATGGTTCTCACTATC	TGCCAAGCCTACAACTGCTA
C. suis ompA	GCTGTTGCCTGTGCTGCAGTAG	GGATACTCCTAGCACTAACA
23S rRNA probe	CGGAGTAAGTTAAGCACGC	TAACGAAAGTTATCTCGCG
Central *rrn* sequencing primers	AGTAAATTCTTTGAGAATCCGCT	GAGCTTCAACTTCTTTTTCCTTGGTTG
Downstream *rrn* sequencing primers	GATAGATGCGTGAAGTATTTTTC	TTGGCTTTTCTTGATCTGTGTG

these recombination events remains to be elucidated. In order to explore possible tools for the development of a workable genetic system for directed mutagenesis of *Chlamydia* DNA, and to investigate the possibility of tetracycline resistance acquisition in human strains of *C. trachomatis*, we conducted in vitro recombination experiments using rifampin (rifampicin)-, ofloxacin-, and tetracycline-resistant strains as donors and recipients. Our results demonstrate that recombination can be readily demonstrated within and among species of the genus *Chlamydia* and that tetracycline resistance can be manifested by *C. trachomatis* following coculture with tetracycline-resistant *C. suis* strains. Tetracycline resistance was not successfully transferred to *Chlamydophila caviae*, suggesting a biological barrier that does not allow recombination across these genera.

MATERIALS AND METHODS

Chlamydial strains, culture, and antibodies. *Chlamydia trachomatis* strains included UW-6276/J/cx, UW-70/F/cx, and L2/434/Bu. Single strains of *C. muridarum* (Nigg) and *Chlamydophila caviae* (GPIC) and two strains of *C. suis*, R19/tet^R and S45/rif^R, were used in the crosses. All strains were propagated from a collection of frozen samples stored at the University of Washington Chlamydia Repository (29). Specimen collection, culture isolation techniques, and serotyping were conducted as described previously (27). Briefly, patient swabs were collected and stored in *Chlamydia* transport medium at 4°C and were transported to the laboratory within 24 h. Each specimen was inoculated onto McCoy cells, centrifuged at 1,200 × *g*, aspirated, and overlaid with minimal essential medium to which 10% fetal bovine serum and cycloheximide (1.0 μg/ml) had been added (MEM-10). Cells were incubated at 37°C under 4% CO_2 for 48 h and were fixed with methanol. Chlamydial growth was detected by fluorescence microscopy using the genus-specific monoclonal antibody (MAb) E6-H1 (a gift from Harlan Caldwell, NIAID). Isolates were then cloned by a twofold dilution method (6). The resulting cloned elementary bodies were grown to high titers and were partially purified by centrifugation of lysates of infected cells through a 30% Renografin pad (4). MAbs specific for major outer membrane proteins (MOMP) of *C. trachomatis* serovars J and F, *C. muridarum* (MoPn), and *Chlamydophila caviae* GPIC, and a polyclonal antibody specific for *C. suis* strains, were used for labeling of cultures coinfected with products of recombinant crosses.

Selection for resistance. Strains of *C. trachomatis*, *C. muridarum*, *Chlamydophila caviae*, and *C. suis* were inoculated onto McCoy cells at a multiplicity of infection of 1.0 in 75-cm^2 flasks, centrifuged by using a Beckman model J-6 M centrifuge at 1,200 × *g* for 1 h at 37°C, overlaid with subinhibitory concentrations (equivalent to half the MIC) of the appropriate drug, and incubated at 37°C for 24 to 48 h, depending on the species inoculated. The cell layers were then disrupted by freeze-thawing at −80°C and 37°C. This was followed by disruption of monolayers by gentle aspiration with a micropipette and low-speed centrifugation to remove debris. Supernatants from disrupted cell layers were passed onto fresh McCoy cell monolayers in 12-mm^2 shell vials to select for resulting mutants as described previously (26). Briefly, serial twofold dilutions of either rifampin or ofloxacin made in MEM-10 were added. The emergence of resistance was monitored using immunofluorescence microscopy following culture in 48-well tissue culture plates. As resistant inclusions emerged, the progeny were cloned by limiting dilution, and their MICs were determined. In one case (see Table 2, cross 5, strain L2/ofl^R-rif^{R}1), a strain was first selected for resistance to ofloxacin, and a cloned ofloxacin-resistant product was then selected for resistance to rifampin.

Recombination experiments. All crosses were performed in sets of eight shell vials (12 mm^2) seeded with 4.0 × 10^5 McCoy cells. The monolayers were then infected with different combinations of drug-resistant strains and species of chlamydiae, each at a multiplicity of infection of approximately 2.0, ensuring infections of cells with both strains. Cultures were incubated from 24 to 48 h postinfection, depending on the species infected, in the absence of antibiotics and were then harvested using the freeze-thaw method described above. Potential recombinants were selected by inoculating 50 μl of the freeze-thaw lysates from each shell vial onto a new shell vial monolayer and overlaying with a medium containing antibiotics at 4 times the MIC for each resistant parental strain (see Table 2). Cultures were grown at 24 to 48 h postinfection, and surviving recombinants were detected by immunofluorescence on parallel monitoring plates. Total supernatants from primary freeze-thaw lysates of negative passages were then inoculated into new vials and cultured in the presence of selecting antibiotics. This technique was modified depending on the rate of recovery of recombinant chlamydiae, with blind passages repeated up to four times in the presence of selecting antibiotics if cultures remained negative. Recovered recombinants were then cloned by limiting twofold dilution, and the species/strain was determined. Analysis of individual cloned strains involved PCR confirmation of *ompA* or *tet*(C) genotypes, gene-specific PCR and sequence analysis, or genome sequencing, as indicated.

Immunofluorescence microscopy of coinfected cultures. To illustrate the cohabitation of various chlamydiae with different drug markers within the same inclusion, McCoy cells were grown on coverslips in 12 mm^2 shell vials and were coinfected with various combinations of rifampin-, ofloxacin-, and tetracycline-resistant chlamydiae. If growth rates were significantly different (i.e., *C. muridarum* and *C. trachomatis* serovar J), infections were staggered to allow the development of each strain in the infection. Cells containing mature inclusions were labeled with monoclonal or polyclonal anti-MOMP antibodies specific for the species or strain used in coinfection and with appropriate secondary antibodies that were labeled either with rhodamine or with fluorescein (Southern Biotech, Birmingham, AL).

***tet*(C) and *ompA* PCR.** PCR was performed using standard protocols and cycling parameters. Primers specific to *tet*(C) (Table 1) were used to screen for tetracycline-resistant recombinants. Primers specific to *ompA* were designed at polymorphic loci of the gene to distinguish between strains (Table 1). References to gene numbers in *C. trachomatis* are based on the L2b/UCH-1/proctitis genome sequence (31) (DDBJ/EMBL/GenBank accession number AM884177), while gene numbers for *C. muridarum* are based on the *C. muridarum* Nigg sequence (22) (DDBJ/EMBL/GenBank accession number AE002160).

Southern blotting. EcoRI-digested genomic DNA was blotted onto a nylon membrane and probed with a digoxigenin-labeled PCR product specific to the 23S rRNA of both *C. trachomatis* L2/ofl^R and *C. suis* R19/tet^R (Roche Diagnostics, Indianapolis, IN) using the oligonucleotide primers shown in Table 1. Probes were incubated overnight and then washed with 0.1% sodium dodecyl sulfate and 10% 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). An alkaline phosphatase-conjugated anti-digoxigenin antibody was incubated with the blot, developed using CSPD substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate and exposed to film.

Illumina paired-end genome sequencing. Purified elementary bodies were incubated with 4 U/ml RQ1 DNase I (Promega) for 60 min, and the DNase was inactivated with 2 mM EGTA. Chlamydial elementary bodies were suspended in a buffer containing 5 mM dithiothreitol, and DNA was extracted using a Qiagen Genomic Tip kit. DNA was further processed for Solexa-based sequence analysis by using commercial DNA preparation kits (Illumina, Inc., San Diego, CA), according to the manufacturer's instructions.

Draft genomes were first assembled using the reference-guided assembly soft-

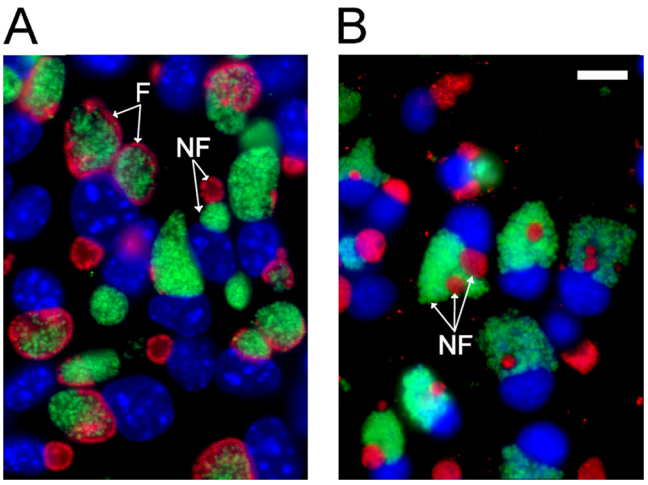

FIG. 1. Immunofluorescence images of McCoy cells coinfected with *C. suis* R19/tet^R and *C. muridarum* MoPn/ofl^R (A) or with *C. suis* R19/tet^R and *Chlamydophila caviae* GPIC/rif^R (B). In both panels, *C. suis* R19/tet^R is labeled red, while the alternate species is labeled green, using species-specific anti-MOMP antibodies. Arrows point to fused inclusions (F) or nonfused inclusions (NF) between the two different infecting strains. Nuclei are labeled blue with 4′,6-diamidino-2-phenylindole (DAPI). Bar, 10 μm.

ware Maq (http://maq.sourceforge.net/). Regions in the reference-guided assembled genome where Maq was not able to resolve the sequence were then compared to contiguous sequences assembled through the use of de novo assembly software (VCAKE [14]), and a single contiguous draft sequence was produced. DNA sequences were compared to the published *C. trachomatis* L2 genome sequence (GenBank accession number AM884177), to draft contigs from the unpublished *C. suis* genome (kindly provided by Garry Myers at the University of Maryland), and to the *tet*(C) island characterized by Dugan et al. (10) (GenBank accession number AY428550). Any necessary manual sequence analysis was performed using MacVector sequence analysis software (MacVector, Cary, NC). rRNA operon (*rrn*) sequences that could not be resolved by de novo assembly of Illumina data were amplified by PCR and subjected to sequence analysis. PCR fragments were generated using primers specific to the genes flanking each *rrn* operon. Primers used to amplify sequences from the central *rrn* operon are shown in Table 1.

Nucleotide sequence accession numbers. The *Chlamydia trachomatis* L2tet1 (L2/tet^{R}1) Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under project accession number ACUI00000000. The version described in this paper is the first version, ACUI01000000. The *Chlamydia muridarum* MopnTet14 (MoPn/tet^{R}14) Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under project accession number ACUJ00000000. The version described in this paper is the first version, ACUJ01000000.

RESULTS

Immunofluorescence images of crossed strains. The immunofluorescent images in Fig. 1A illustrate the fusion of *C. suis* R19/tet^R and *C. muridarum* MoPn/ofl^R inclusions during coinfection in the course of recombination experiments. In these images, *C. suis* R19/tet^R developmental forms are labeled red and rifampin- or ofloxacin-resistant developmental forms are labeled green. These experiments demonstrate that *C. muridarum* MoPn/ofl^R and *C. suis* R19/tet^R form fusogenic inclusions in culture. Crosses between different strains of *C. trachomatis* and either of these strains yielded results similar to those in previously published work (29, 31). In contrast, none of the tested *Chlamydia* spp. formed fusogenic inclusions with *Chlamydophila caviae* (Fig. 1B).

Demonstration of transfer of rifampin and ofloxacin resistances within *Chlamydia* spp. Rifampin-, ofloxacin-, and tetracycline-resistant strains were either generated in this study or generated or described in previous work (25) and were used as primary parental strains in these crosses. Experiments were first designed to replicate the results of Demars et al., who showed that chlamydiae can exchange antibiotic resistance markers (7). Intraspecies and interspecies crosses between rifampin-resistant and ofloxacin-resistant strains of *C. trachomatis*, *C. muridarum* MoPn/ofl^R, or *C. suis* S45/rif^R generated recombinants containing *ompA* from one parent and the antibiotic resistance profiles of both parents (Table 2, crosses 1 to 4). These crosses show that parental antibiotic resistance profiles are reflected in the resistance of the progeny. For example, in cross 1, the rifampin MIC for the *C. suis* S45/rif^R parent is 0.25 μg/ml, and the ofloxacin MIC for the *C. trachomatis* L2/ofl^R parent is 16 μg/ml. The progeny strain shares each of these MICs. Nucleotide sequence analysis of the *rpoB* and *ompA* PCR products of this cross showed that the clone possessed the L2 *ompA* and the S45 *rpoB* sequence (data not shown). The results shown in cross 2 demonstrated that recombination occurs in crosses where one parent is a recent clinical isolate of *C. trachomatis*. Sequencing of the *gyrA* and *ompA* PCR products amplified from this cross demonstrated that the cloned progeny carried serovar J *ompA* and the serovar L2 *gyrA* sequence (data not shown). These data confirm the work of Demars et al. (6, 7) and demonstrate that crosses can be conducted between different species of *Chlamydia*.

Mobilization of tetracycline resistance among chlamydial strains. Table 2, crosses 5 to 10, describes the transfer of tetracycline resistance in crosses between a tetracycline-resistant parent and a rifampin- or ofloxacin-resistant parent, within and between different species of *Chlamydia*. In all cases, PCR was used to demonstrate that the progeny strains carried *tet*(C) and had an appropriate *ompA* genotype. These PCR results also confirmed that DNA from only one parental strain was detectable in the cloned progeny. For example, in cross 5, the PCR for serovar L2 *ompA* was positive but the PCR for *C. suis* R19 *ompA* was negative (Fig. 2). The *tet*(C) coding sequences in the *C. suis* donor and the cloned recombinant progeny were amplified. Similar sets of PCRs were used to confirm each of the cloned progeny shown in Table 2 (data not shown).

Serial transfer of tetracycline resistance is shown in crosses 7, 8, 9, and 10, where a recombinant progeny from one cross was used as a donor in a subsequent cross. For example, the product of cross 7, L2/tet^{R}13, is a tetracycline-resistant clone that has the L2 MOMP. This strain was used as a parent in cross 8, yielding a progeny strain with tetracycline resistance from the R19/tet^R parent in cross 7 and rifampin resistance and MOMP sequences from the serovar J parent. These results demonstrate that transfer of *tet*(C) can occur between each of these species and that a recipient from one cross can serve as a donor in a subsequent cross. Cross 10 shows *tet*(C) transfer between two recent *C. trachomatis* clinical strains, demonstrating that the tetracycline-resistant phenotype can be mobilized among clinical strains of *C. trachomatis*.

The generation of recombinants was limited to crosses within the genus *Chlamydia*. No conditions could be established to generate doubly resistant progeny from crosses be-

TABLE 2. MICs of rifampin, ofloxacin, and tetracycline, and MOMP phenotypes, for each parent and progeny strain[a] in recombinant crosses

Cross	Parental strain	Progeny strain	MOMP phenotype	MIC (μg/ml) of:		
				Rifampin	Ofloxacin	Tetracycline
1	L2/ofl^R		L2	0.004	16	0.032
	S45/rif^R		*C. suis*	0.25	0.5	0.032
		L2/ofl^R-rif^{R}2	L2	0.25	16	0.032
2	L2/ofl^R		L2	0.004	16	0.032
	J/6276/rif^R		J	8	0.5	0.032
		J/6276/ofl^R-rif^{R}1	J	8	16	0.032
3	MoPn/ofl^R		MoPn	0.008	32	0.032
	S45/rif^R		*C. suis*	0.25	0.5	0.032
		MoPn/ofl^R-rif^R	MoPn	0.25	32	0.032
4	MoPn/ofl^R		MoPn	0.008	32	0.032
	J/6276/rif^R		J	8	0.5	0.032
		J/6276/ofl^R-rif^{R}2	J	8	32	0.032
5	L2/ofl^R-rif^{R}1		L2	128	16	0.008
	R19/tet^R		*C. suis*	0.004	0.5	8
		L2/tet^{R}1	L2	128	16	8
6	MoPn/rif^{R}ofl^R		MoPn	128	32	0.008
	R19/tet^R		*C. suis*	0.004	0.5	8
		MoPn/tet^{R}14	MoPn	128	32	8
7	L2/ofl^R		L2	0.004	16	0.032
	R19/tet^R		*C. suis*	0.004	0.5	8
		L2/tet^{R}13	L2	0.004	16	8
8	**L2/tet^{R}13**		L2	0.004	16	8
	J/6276/rif^R		J	8	0.5	0.032
		J/6276/tet^R	J	8	0.5	8
9	MoPn/ofl^R		MoPn	0.008	32	0.032
	J/6276/tet^R		J	8	0.5	8
		MoPn/tet^{R}1	MoPn	0.008	32	8
10	**J/6276/tet^R**		J	8	0.5	8
	F/70/rif^R		F	32	0.5	0.032
		F/70/rif^R-tet^R	F	32	0.5	8
11	L2/ofl^R		L2	0.004	16	0.032
	GPIC/rif^R		GPIC	16	0.5	0.032
		None				
12	R19/tet^R		*C. suis*	0.004	0.5	8
	GPIC/rif^R		GPIC	16	0.5	0.032
		None				

[a] Progeny strains that are used as parental strains in subsequent crosses are shown in boldface.

tween *C. suis* R19/tet^R or *C. trachomatis* L2/ofl^R and the more distantly related strain *Chlamydophila caviae* GPIC/rif^R (Table 2, crosses 11 and 12). This included 64 independent wells for each attempted cross.

Recombination targets the downstream *rrn* operon of the L2 recipient genome. *Chlamydia* spp. share highly similar genetic profiles in regions surrounding the paired rRNA operons (*rrn*). These operons are composed of a 5′ 16S RNA (1.55 kb), a central 23S RNA (2.87 kb), and a 3′ 5S RNA (116 bp) (see Fig. 5). The two *rrn* operons are separated by ~26,000 bp of DNA in *C. trachomatis* L2/ofl^R and *C. muridarum* MoPn/ofl^R and by ~35,000 bp in *C. suis* R19/tet^R. The difference in size between these strains is due to the presence of the *tet*(C) genomic island, which is integrated into the *inv*-like gene of R19 and other tetracycline-resistant *C. suis* strains. This island lies just downstream of the first *rrn* operon (10) (see Fig. 4). The recombination shown in cross 5 between the tetracycline-resistant *C. suis* strain R19 and a rifampin-resistant *C. trachomatis* L2 progeny strain occurred in this region of the chromosome. Southern blotting with a common rRNA probe confirmed the presence of the 23S rRNA genes of both *C. suis* R19/tet^R and *C. trachomatis* L2/ofl^R in the recombinant (Fig. 3). While these Southern blots showed that cloned recombinants carried ribosomal sequences from both parents, PCR analysis demonstrated that the recombinant did not have *C. suis ompA* DNA (Fig. 2). Genome sequence analysis of the progeny from cross 5, L2/tet^{R}1, demonstrated that a ~40-kb fragment of donor DNA recombined into the *C. trachomatis* L2/ofl^R-rif^R recipient

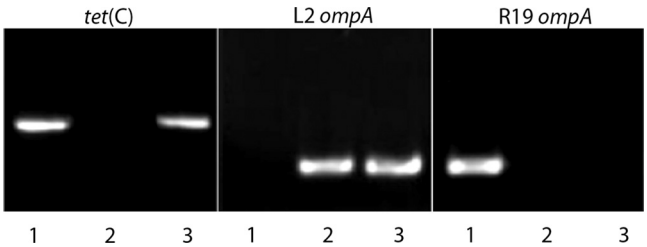

FIG. 2. PCR results for parental strains R19/tet^R (lanes 1) and L2/ofl^R-rif^{R}1 (lanes 2) and for the cloned L2/tet^{R}1 recombinant (lanes 3). Primers are specific to *tet*(C) (left), to *ompA* from parental strain L2/ofl^R-rif^R (middle), and to *ompA* from parental strain R19/tet^R (right), The molecular size of the *tet*(C) PCR product is 525 bp, and those of the *ompA* PCR products are 248 bp for L2/ofl^R and 258 bp for R19/tet^R.

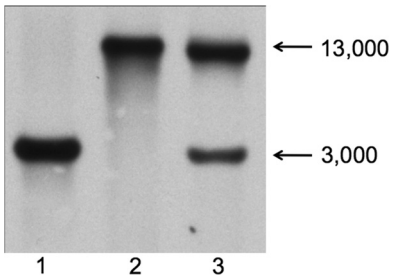

FIG. 3. Southern blotting of EcoRI-digested genomic DNAs from parental and recombinant strains (Table 2, cross 5). Digested genomic DNAs were hybridized with DNA probes specific to the donor (R19/tet^R) and recipient (L2/ofl^R-rif^{R}1) 23S rRNA sequences. Lane 1, L2/ofl^R DNA, showing a doublet of two bands (3,024 and 2,973 bp); lane 2, R19/tet^R DNA, showing a predicted band of 13,546 bp; lane 3, cloned L2/tet^{R}1 progeny from the cross of these two strains, showing appropriately sized fragments of both parental 23S rRNAs. The approximate numbers of base pairs in the observed bands are given on the right.

strain, leading to a merodiploid structure with three *rrn* operons (Fig. 4). The transferred DNA contained the *tet*(C) island along with 10 neighboring *C. suis* genes (homologs of CTLon_0109 to CTLon_0118), flanked by the two *rrn* operons. The upstream (L2 parental) *rrn* operon was not involved in the recombination. The sequencing of the two downstream *rrn* operons, however, provides evidence for the integration of *C. suis* DNA into the recipient, yielding hybrid sequences containing evidence of both parental strains (Fig. 5). The upstream recombination site is within the 23S rRNA gene sequence in the central *rrn* operon, where the sequence transitions from recipient L2/ofl^R-rif^R DNA to donor R19/tet^R DNA within a 75-bp stretch of identical nucleotides. The downstream *rrn* operon is a mosaic containing seven different recombination sites (Fig. 3 and 5). The first crossover site in this *rrn* operon lies just upstream of the 16S rRNA gene sequence in the intergenic region between CTLon_0118 and the 16S rRNA. This is followed by two more transitions in the 16S rRNA gene, a single transition in the 16S–23S intergenic region, and three more in the 23S rRNA gene. No insertions or deletions of *rrn* DNA were observed, and thus, the overall size and structure were conserved. This recombination created three potentially functional sets of rRNA clusters within the progeny strain.

Homologous-recombination-mediated transfer of tetracycline resistance into *C. muridarum*. Sequence analysis of the MoPn/tet^{R}14 recombinant (Table 2, cross 6) demonstrates a

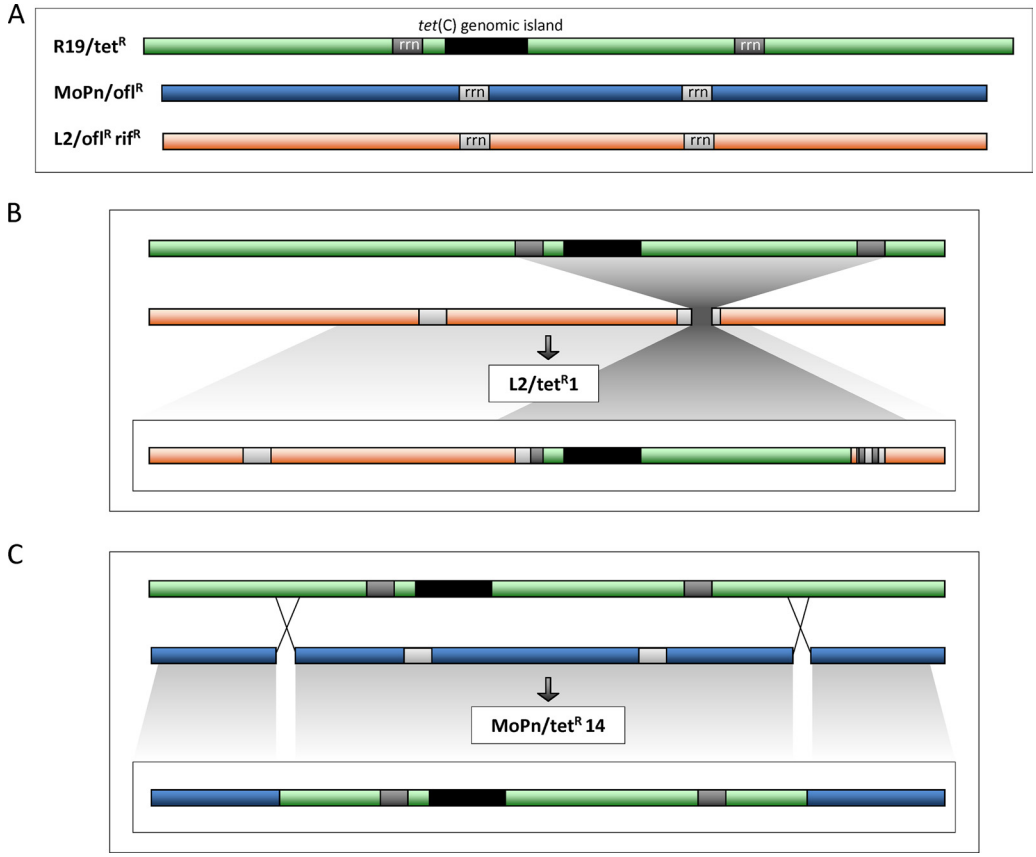

FIG. 4. Recombination models for L2/tet^{R}1 and MoPn/tet^{R}14. (A) Parental strains, showing the locations of ribosomal operons (*rrn*) and the *tet*(C) genomic island. (B) *C. suis* R19/tet^R donor DNA integrated at the downstream 23S rRNA of *C. trachomatis* L2/ofl^R-rif^{R}1, producing a recombinant with three *rrn* operons and ~40 kb of donor DNA. The two downstream *rrn* operons are hybrid genes, each composed of a mosaic of donor and recipient parental DNAs. (C) *C. suis* R19/tet^R donor DNA recombined at two loci outside of the *rrn* sequences. The MoPn/tet^{R}14 recombinant has *C. suis* R19/tet^R copies of genes TC_0081 through TC_0149, which include the intact *C. suis* R19/tet^R *rrn* operons and the *tet*(C) genomic island. The remainder of MoPn/tet^{R}14 is identical to the other parental strain, *C. muridarum* MoPn/ofl^R.

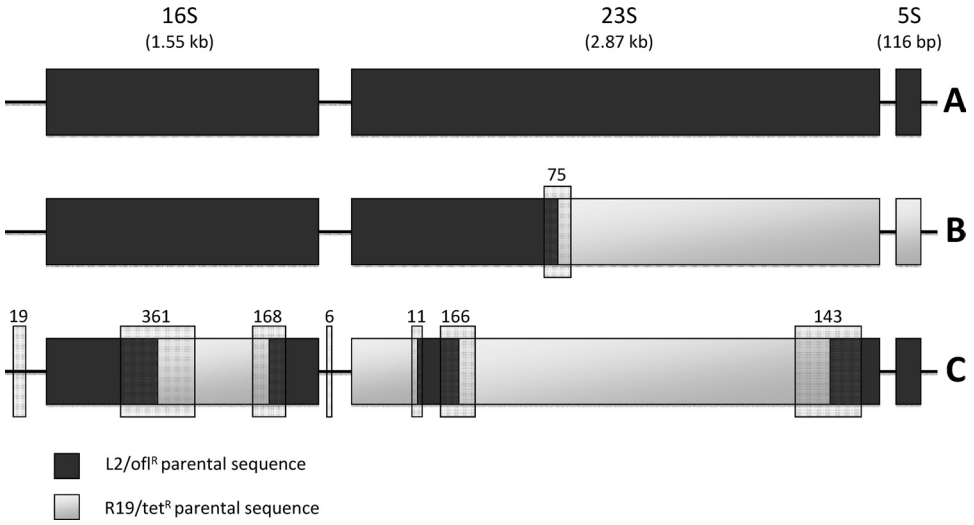

FIG. 5. Ribosomal operons and recombination sites in the sequenced recombinant L2/tet^{R}1. (A) The leftmost rRNA operon is completely derived from the parental L2 sequence. (B) The central rRNA operon (see Fig. 4) is a hybrid of the two parental genomes, with a single crossover point identified within the 23S gene. (C) The rightmost rRNA operon is a mosaic of the two parental sequences. The source of each sequence in the L2/tet^{R}1 genome (the recipient strain, *C. trachomatis* L2/ofl^R-rif^{R}1, or the donor strain, *C. suis* R19/tet^R) is indicated. Identical stretches of DNA sequence in the two parental strains, where the crossovers occurred, are boxed. The lengths (in base pairs) of the identical sequences at the recombination sites are given above each box.

product that is structurally different from that observed in L2/tet^{R}1. Although the two parental genomes share features similar to those observed for *C. suis* R19/tet^R and *C. trachomatis* L2/ofl^R (95.2% identity at *rrn* loci for *C. suis* R19/tet^R and *C. muridarum* MoPn/ofl^R), the DNA acquired in the cross was the result of a classical double-crossover event that lacked the mosaicism or the merodiploid structure identified in the *C. trachomatis* L2/tet^{R}1 clone (Fig. 3 to 5). Instead, a 98,375-bp DNA fragment containing 68 open reading frames, 2 *rrn* operons, and the *tet*(C) island recombined into the *C. muridarum* MoPn/ofl^R recipient genome, displacing approximately the same amount and functional content of *C. muridarum* MoPn/ofl^R DNA (Fig. 4). A stretch of 17 nucleotides (CCTCCAGT TTCTGAAATT) that is identical in the two parental genomes marks the upstream recombination site inside TC_0081 (encoding a helicase in the Snf2 family). The downstream recombination event occurred within a stretch of 10 identical nucleotides (TTGGAAGACGA) inside TC_0149 (encoding a hypothetical protein). The resulting recombinant is a hybrid of the two species, consisting of approximately 9.1% *C. suis* R19/tet^R DNA and 90.9% *C. muridarum* MoPn/ofl^R DNA.

DISCUSSION

Demars and colleagues demonstrated in vitro horizontal gene transfer in *C. trachomatis* by coinfecting cell cultures with two strains and selecting for horizontal gene transfer through the use of appropriate antibiotics (6, 7). In their work, *C. trachomatis* L1 strains harboring mutations in *gyrA* or *rpoB* were used as donors and recipients in coinfections. Doubly resistant progeny were screened for the presence of these two markers and were then further characterized on the basis of genotypes at several polymorphic loci across the genome. The estimated size of the DNA transferred was between 123 kb and 790 kb based on the genotyping approach used. Two of the recombinants analyzed carried insertions of DNA in the target genes *rpoB* and *gyrA*. Our studies confirm these findings and expand the results of Demars and colleagues by demonstrating rifampin and ofloxacin resistance transfer within and among chlamydial species (Table 2, crosses 1 to 4). Our results also demonstrate that a naturally occurring tetracycline resistance marker from *C. suis* can be transferred within a species, between species, and into recent clinical isolates. The genome sequences of two different recombinants were completed in order to determine exact recombination sites and to evaluate the mechanistic details of the DNA fragments recombined into the recombinant genomes. The fully completed genome sequences from L2/tet^{R}1 and MoPn/tet^{R}14 provide considerable information about the nature of recombination in this system. There is evidence of both insertional and replacement recombination that leads to the incorporation of a broad range of sizes of DNA into the target genome. This is consistent with the work of Demars et al., who conducted PCR-based mapping experiments and showed surprisingly large insertions and replacements in their tested genomes (6, 7). While insertion of DNA into a chromosome suggests a classical single-crossover event and a replacement of DNA generally is associated with a double crossover, the assembly of the complete genome sequences reported here demonstrated that each event involved at least two recombination events. This was represented by parental mosaicism at chlamydial recombination sites in the *C. trachomatis* L2/ofl^R-rif^{R}1 × *C. suis* R19/tet^R cross (Fig. 3 to 5).

In our experiments, recombination between *Chlamydia* spp. was regularly documented, but we were not able to produce recombinants in a cross between *Chlamydophila caviae* GPIC/rif^R and either *C. suis* R19/tet^R or *C. trachomatis* L2/ofl^R-rif^{R}1. There are several possibilities that may account for our inability to recover such crosses. First, it is possible that *Chlamydophila caviae* strains are not actively recombinogenic. Second,

the level of sequence identity at possible recombination sites may have not been adequate. Finally, it is possible that recombination requires occupation of a common vacuole or perhaps unique molecular interactions, and the *Chlamydia* spp. demonstrated to be recombinogenic cannot achieve the required recombinogenic state with *Chlamydophila caviae* GPIC/rif^R. While the latter possibility remains a logical option, preliminary work in our laboratories indicates that IncA-negative, nonfusogenic strains of *C. trachomatis* can recombine with wild-type strains, suggesting that fusion of inclusions is not required in order for recombination to occur (R. J. Suchland, unpublished data). These issues remain to be elucidated as we work to identify the recombination mechanisms used by *Chlamydia* spp.

Stable antibiotic resistance in isolates from human chlamydial infections has not yet been substantiated. While there have been numerous reports of patients with apparent treatment failure, these strains usually exhibit a heterotypic pattern of resistance, with only a small proportion of the population of organisms surviving after exposure to antibiotics (15, 24). The absence of antibiotic resistance is consistent with the fact that chlamydiae in general do not reflect acquisition of foreign DNA in the recent evolutionary past. The relative lack of genomic islands within these organisms has led to the supposition that recombination in the chlamydiae is a very rare event. However, genomic studies, such as those conducted by Dean and colleagues (11, 12), and the pioneering work of Demars et al. (6, 7) have suggested that interchlamydial recombination does occur and perhaps is common. Our work supports this model.

The two sequenced genomes presented here provide few clues as to potential target sequences, target sizes, or any sequence specificity that may be important in chlamydial recombination. The recombination sites differ considerably in size and content, ranging from a very short, 11-bp sequence between *C. muridarum* MoPn/ofl^R and *C. suis* R19/tet^R to a much larger, 366-bp stretch of DNA between *C. suis* R19/tet^R and *C. trachomatis* L2/ofl^R-rif^{R}1 in the L2/tet^{R}1 recombinant. The sequence identity at the *rrn* operons guided the L2/tet^{R}1 recombination, but this was not the target for recombination in the MoPn/tet^{R}14 clone. The apparent lack of uniformity between recombination sites in these genomes provides evidence in favor of a highly versatile recombination mechanism that permits genomewide transfer of chlamydial DNA at any appropriately targeted loci. Additional sequencing of recombinant progeny from a variety of intraspecies and interspecies crosses will provide more data for the study of these unknown features of recombination in chlamydiae.

It is estimated that active trachoma, caused by ocular strains of *C. trachomatis*, affects an estimated 84 million people, of whom about 1.3 million are blind (20). The introduction and spread of tetracycline resistance into trachoma-causing strains would cause major worldwide public health concerns, because tetracycline and its derivatives are critical therapeutic agents in the fight against these infections, particularly in the developing world. Furthermore, millions of patients with sexually transmitted chlamydial infections would also be affected by chlamydial tetracycline resistance. Our work demonstrates that if a naturally occurring *C. trachomatis* strain acquired a *tet*(C) resistance allele, cross-serovar transmission through a patient population treated with tetracycline might occur, perhaps rapidly. Dissemination of *tet*(C)-carrying *C. suis* strains has already been observed in pig populations—another setting that relies heavily on tetracycline for treatment and prophylaxis.

These recombination experiments challenge the premise that significant antibiotic resistance problems will not be observed in *C. trachomatis* and present a model for how such transfers may occur. At present, pig populations in the Midwestern United States are commonly infected with *C. suis*, and screening suggests that a high percentage of these strains are tetracycline resistant (1, 5; A. Andersen, personal communication). Resistant strains were also described recently in Italy (8). *C. suis* strains carrying the different *tet*(C) islands have different MOMP sequences, suggesting that they are not clonal in origin (3). Dugan et al. (10) identified four different genomic islands in the strains isolated from the United States, each of which contains 3 to 10 kb of DNA that is very nearly identical to plasmid pRAS3.2, carried by the gram-negative fish-pathogenic bacterium *Aeromonas salmonicida* (16). Plasmid pRAS3.2 does not, however, carry IS*cs605*, the IS element present in a majority of the *tet*(C) islands, which has been found only in resistant *C. suis* strains. We hypothesize that the plasmid entered the pig digestive tract in *A. salmonicida* or some other gram-negative bacterium, possibly via a feed source. Considering that IS elements related to IS*cs605* are found in *Helicobacter* spp. (13), the plasmid may have picked up the IS element during passage in the porcine gastrointestinal tract. The complete genomic island was then physically delivered to *C. suis* via a completely uncharacterized process. Pigs are routinely fed tetracycline during growth in production facilities, and therefore the ability to grow in low levels of this drug remains a major selective force for porcine commensals and pathogens. The entry of the resistance gene into the population may have led to rapid clonal proliferation within an unexploited niche, followed by homologous recombination between different *C. suis* strains, and likely at least some activity by the transposase encoded by IS*cs605*, to generate the family of *tet*(C) islands currently found in *C. suis* (9). The overriding hypothesis in this scenario is that the entry of the genomic island into *C. suis* was initially challenging in this natural system, but transfer among strains, via a homologous-recombination process similar to that demonstrated in this report, was straightforward and perhaps rapid. Our studies demonstrate that transfer of the marker from *C. suis* to other, related chlamydiae in the laboratory is routine if the strains grow within the same cell culture environment. Therefore, contact between tetracycline-resistant and tetracycline-sensitive *Chlamydia* spp. in any setting may lead to transfer of the resistance genes and the resulting phenotype, which could then be propagated and selected for in patients treated with tetracycline.

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